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AD

FOLLOW-UP STUDIES OF TICK-BORNE ENCEPHALITIS

(Ecological, epidemiological and experimental laboratory studies).

Final Technical Report

By

Ch.Kunz,M.D., W.Frisch,Ph.D., A.Radda,Ph.D.,
G.Pretzmann,Ph.D.

March 1967

EUROPEAN RESEARCH OFFICE

Contract Number 91-591-EUC-4031

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Hygiene-Institut
der Universität
Kinderspitalgasse 15
Wien 9, Austria

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A b s t r a c t

In field studies 27 virus strains were isolated from ticks.

A focus of TBE virus was found considerably further west than any other focus previously detected.

Sera of small mammals showed a low incidence and sera of game showed a high incidence of antibodies to TBE virus. Virus strains could be rapidly identified with fluorescent antibodies. The innate resistance to TBE virus of Mus musculus spicilegus is probably due to a single dominant gene. The mouse brain lipid capable of inhibiting hemagglutination of TBE virus is most probably phosphatydil-inositol-diphosphate. Its full action requires the presence of auxiliary lipids containing a trimethyl-ammonium group. Preliminary results indicate that the lipid can also inhibit the virus adsorption onto tissue culture cells.

In sera from both the acute stage of TBE and from convalescence complement-fixing antibodies were predominantly found in the 7 S γ-globulin fraction.

From 39 persons 38 still had complement-fixing and all had hemagglutination-inhibiting antibodies 6-9 years after overt TBE.

Continuous propagation of a TBE virus strain led to overattenuation of the virus. In 1966, 136 TBE cases from Austria and 5 cases from Western Germany were diagnosed.

C O N T E N T S

FIELD STUDIES

(1) Introduction

In 1965, field studies on the ecology of TBE virus were carried out in 4 areas (Gfieder, Strelzhof, Hernstein and Leithagebirge) in the eastern part of Austria. Virus isolations were successful only from ticks collected in Strelzhof and Hernstein. The role of mice in the virus cycle in nature was studied. The results of these investigations have led to the assumption that the high number of infected ticks observed in 5 areas covering only a few square feet (microfoci) is due to other mammals which are larger than mice (1,3).

In Hernstein and in Strelzhof, where the virus has been active in the last year, tick collections and mark-and-release trapping of mice were carried out at regular intervals during 1966. On the Gfieder, ticks were collected only once for virus isolation experiments. In addition, two locations near Linz (Upper Austria) were searched for ticks. Persons who later developed TBE had visited these locations before becoming ill. In order to clarify further the role of the different species of mammals in the cycle, mass collections of small mammals in Hernstein were carried out, and blood samples of game were tested for antibodies against TBE virus. It was also planned to include hedgehogs in this study. However, in Hernstein and Strelzhof no hedgehogs could be found (according to the forest officials, hedgehogs do not occur in the areas). Therefore, field studies were carried out in the steppe habitats to the east of the Neusiedlersee (near the small village of Apetlon) where hedgehogs are relatively abundant.

(2) Areas under investigation

(a) Hernstein (Lower Austria), 500 m above sea level. Limestone and pontic conglomerate. Pinewood forest (Pinus nigra) containing forest clearings with brushwood (Carpinus betulus, Corylus avellana, Cornus spp. a.o.). (A map of this area is given in last year's report (1).

(b) Strelzhof (Lower Austria), 400 m above sea level. Trias-lime. Shrubby Oak-Hornbeam wood with black fir trees (see report of 1965,(2)).

(c) Gfieder (Lower Austria), 550 m above sea level. Quartzey minerals cause acid soils. Pinewood forestation (Pinus sylvestris, Picea excelsea) in an old conifer forest.

(d) Seewinkel near Apetlon (Burgenland), 200 m above sea level. Solonetz and Solotschak-soils. Steppe habitats with small forestations.

(e) St.Florian and Pulgarn near Linz (Upper Austria), about 260 m above sea level. Alluvial gravels. Mixed pinewood forestations with Larix decidua and partially dense undergrowth (Rubus spp.).

(3) Tick collections and virus isolations

Methods: In the Hernstein and Strelzhof areas, 16 marked points were searched for ticks at regular time intervals. In the Gfieder area ticks were collected only once in June. In the foci near Linz, ticks were collected twice at random, one in June and again in September. Only nymphs and adults were collected and transported to the laboratory. The nymphs were homogenized in pools of 20 individuals (50 individuals in the case of nymphs from Upper Austria), suspended in a medium consisting of PBS and 10 % calf serum and inoculated i.c.into baby mice. The adults were treated in the same manner, but pool size varied from 1 to 5 individuals in the case of ticks from Upper Austria.

Results: In Table 1, time and results of tick collections, number of tested pools and number of isolated strains of virus can be seen. A total of 1279 ticks were collected in Hernstein and tested for virus in 73 pools. Five strains of TBE virus could be isolated. From 2103 ticks collected in Strelzhof and tested in 146 pools, 6 virus strains were isolated. In the Gfieder area, no virus could be detected. From 2253 ticks collected in St.Florian and Pulgarn near Linz, Upper Austria, 16 virus strains could be isolated. It is striking that all strains isolated in a certain focus originated from ticks which were col-

lected at the same time (Table 2). Out of 6 strains, isolated from nymphs collected in Strelzhof, 4 derived from ticks collected in field 13 and one from ticks collected in field 18. Many virus strains were isolated from this field last year.

(4) Bionomics of mice in relation to the virus cycle in nature

Material and methods: In an area of about 1 hectar small mammal traps were set up about 15 meters from each other. Thirty-six trapping spots in the Hernstein area, and 41 in the Strelzhof area were chosen. Two traps were exposed on each trapping spot. Throughout the period of investigation, traps were baited with sunflower seeds, but most of the time the trapping mechanism was blocked.

At intervals of about 4 weeks (the dates and results of the several excursions are listed in Tables 3 and 6), the traps were additionally baited with carrots and peanut butter and triggered. Traps were inspected in the morning, and captured mice were narcotized in a plastic bag and investigated. Newly captured mice were marked individually by clipping of certain toes. Registration number, species, sex, weight, approximate age and infestation with parasites were recorded; and finally the animals were released at the point of their capture. Those mice which were trapped during the two last excursions were transported to the laboratory in order to take blood from the orbital sinus. The sera of these blood samples were tested by the neutralization test (NT) for antibodies against TBE virus.

Results: (Hernstein). A total of 105 mice was registered in 212 trappings; of these, 14 had been registered in 1965. Table 3 shows the results of mark-and-release trappings of the single excursions. Table 4 shows the abundance of several species. There are no differences in the relative abundance compared with the results obtained in the previous year. The number of individuals registered in 1966 was however decidedly lower than that of the previous year, which may be traced back partially to the larger intervals between the single excursions. Compared to 1965, however, the population densities of all species were lower than

could be expected. Only in October did the population density of the Bank Vole reach about the same level as in 1965. No differences could be observed in the free range life time of mice and their species distribution according to habitat.

In spring the degree of parasitation of mice with ticks was lower than during the months of earlier findings (Table 5). The results of the serological survey of sera from mice are included in these investigations, the results of which are described in the next chapter (under (5)).

(Strelzhof). In Table 6, dates and results of the excursions are listed: 79 specimens could be registered in 111 trappings. This year the predominant species was again the Yellow-necked Fieldmouse (Table 7). Until September, the population density of this species was very low. An immigration of a high number of individuals could be observed in the autumn. This may be traced back to abundant fruiting of oaks within the area during this year.

In Strelzhof, extraordinarily high parasitation of mice by larvae of ticks could be observed by the end of May (Table 8). From 25 blood samples of mice, neutralizing antibodies against TBE virus could be demonstrated in 3 sera of specimens of Clethrionomys glareolus (Table 9).

(5) Serological investigations on small mammals and game from Hernstein and its surroundings

Material and Methods: From April 3-12, from October 4-27, and from November 28-30, mass collections of small mammals were carried out in different spots surrounding Hernstein. For this purpose, about 100 small mammal live traps were set up in different habitats. Traps were baited with carrots and cheese or carrots and peanut butter, and inspected at least twice daily. Blood was taken from the orbital sinus of captured animals. Sera were tested in the NT for antibodies against TBE virus. Virus isolation experiments were done only from blood samples taken from 18 individuals caught in the spring.

During the last excursion at the end of November, Dr.J.Nosek and Dr.O.Kozuch from the Institute of Virology of the Czechoslovak Academy of Sciences in Bratislava spent several days with us in Hernstein. These workers, who have reason to assume that moles and shrews play a major role for the virus cycle in nature (4,5), demonstrated the methods currently used in Czechoslovakia for trapping insectivors. We learned their technique for trapping moles and shrews. In addition, we obtained blood samples from game for serological investigations.

Results: In Table 1o, results of serological studies on small mammals and game are shown. In more than 1,ooo trap units (number of traps times number of nights), only 28 small mammals were caught in the spring, thus indicating that population densities were low at this time of the year. Nevertheless 2 Bank Voles showed antibodies. No virus could be isolated from blood. In the fall, however, in about 15oo trap units, 354 small mammals were caught and bled. Included in this number are blood samples which had been taken from all individuals which were found moribund or dead in the traps and used for mark-and-release trapping throughout the year (about 2o sera). Four Bank Voles, 1 Common Vole and 1 Common Shrew showed antibodies to TBE virus. In about 4o trap units (special traps for moles), only 1 mole was caught, indicating low population density of moles in Hernstein. In the course of an excursion to the Strelzhof focus, evidence was obtained concerning the activity of moles in this area. As regards the tested sera of game, half of the sera of roe deer and of fox were positive in the neutralization test (Table 1o).

(6) Serological investigations on small mammals and game collected in the Seewinkel

The aim of this study was to find out whether or not TBE virus is prevalent in this area which is on the western edge of the Hungarian lowlands. For this purpose hedgehogs, ground squirrels and hamsters were caught. Blood was taken by heart puncture. We also obtained blood samples of game. Altogether 238 sera were tested for TBE antibodies. In Table 11, the numbers of tested sera

of several species are listed. None of the sera gave a clear positive result in the neutralization test. Thus, the area bordering Hungary does not appear to be infested with TBE virus.

(7) Conclusions and Recommendations

The results of our serological investigations have clearly revealed that roe deer and fox, although tested so far only in small numbers, show much higher infection rates than small mammals. This confirms our previous assumption, that mice do play an important role as hosts of tick larvae, but that they do not represent the only essential link in the natural cycle of TBE virus. Hedgehogs could not be found in the foci in Lower Austria. In this type of natural focus, therefore, the abundant occurrence of infected ticks in certain points is due to mammals other than hedgehogs. Such "microfoci" could be detected again this year.

The verification of a focus of TBE in Upper Austria is of considerable interest: 16 virus strains could be isolated from ticks collected in a forest in St.Florian near Linz. To our knowledge, no TBE virus was isolated in Europe further west than this point.

According to the results of serological investigations, carried out in the Seewinkel, natural foci of TBE virus apparently do not occur in this part of eastern Austria.

In future studies participation of game within the virus cycle should be investigated by larger serological surveys. In addition, moles in Strelzhof and shrews in Hernstein should be carefully studied with respect to their role in the virus cycle.

In order to be certain that the Hernstein and Strelzhof areas are still active foci, ticks will be collected and virus isolations will be attempted.

Summary

Field studies on the ecology of TBE virus were carried out in 5 areas (Hernstein, Strelzhof, Gfieder, Seewinkel, St.Florian and Pulgarn near Linz).

Virus was isolated from ticks collected in Hernstein and Strelzhof. No virus could be detected in the Gfieder area. By isolating 16 virus strains, a new focus of TBE virus was found in a forest in St.Florian east of Linz (Upper Austria).

In Hernstein population densities of mice were low in the spring. Relative abundance of several species was the same as in the previous year. Migrations of mice were probably influenced mainly by trophic conditions. Infestation of mice with ticks was not so high as usual in Hernstein. Parasitation of mice in Strelzhof coincided with parasitation observed in the past year.

A low incidence of neutralizing antibodies to TBE virus was found in sera of 382 small mammals thus indicating that these animals are not the only essential vertebrate host for the virus. By contrast, in a few sera collected from roe deer and foxes, a high rate of NT positives was obtained.

No hedgehogs were found in Hernstein and in Strelzhof.

More than two hundred sera from different species of mammals which were caught or shot in the Seewinkel area near the Hungarian border gave no evidence of antibodies against TBE virus.

Table 1: Number of ticks (Ixodes ricinus) collected in different areas and virus strains isolated therefrom.

a) Hernstein

Excursion date	Nymphs	Number of strains isolated	Adults	Number of strains isolated
1 March 19-2o	32 (2)+)	-	6 (2)+)	-
2 April 23-24	388 (17)	-	17 (4)	-
3 May 23-24	339 (18)	2	12 (3)	3
4 June 12-13	351 (17)	-	15 (2)	-
5 Sept. 3-4	55 (3)	-	3 (1)	-
6 Oct. 1-2	60 (3)	-	1 (1)	-
Total	1225 (6o)	2	54 (13)	3
b) Strelzhof				
1 March 2o-21	91 (5)	-	25 (6)	-
2 March 27-28	128 (6)	-	14 (4)	-
3 May 7-8	697 (35)	-	78 (19) + 4 H.c.++)	-
4 May 28-29	527 (27)	6	46 (18)	-
5 Sept. 2o-21	222 (11)	-	3 (1)	-
6 Oct. 14-15	271 (13)	-	1 (1) + 1 H.c.++)	-
Total	1936 (97)	6	167 (49)	-
c) Gfieder				
1 June 9-11	212 (1o)	-	24 (4)	-
d) St.Florian near Linz				
+++) 1 June 1-3	958 (18)	9	1o2 (11)	6
2 Sept. 2o-21	32o (26)	-	32 (-)	1
Pulgarn near Linz				
1 June 1-3	796 (16)	-	45 (5)	-

+) in brackets ( ) number of pools
++) Haemaphysalis concinna
+++) see chapter EXPERIMENTAL LABORATORY INVESTIGATIONS (2)

Table 2: Virus isolations from ticks collected in the foci under investigation in 1966.

Date	Strain No.	Pool	Passage	Focus and field of tick collection
May 23-24	Ix 12183	2o Ny	1	Hernstein 1o,11
" 23-24	Ix 12185	2o Ny	1	Hernstein 14,15,16
" 23-24	Ix 12186	5 F	1	Hernstein
" 23-24	Ix 12187	6 M	1	Hernstein
" 23-24	Ix 12188	1 F	1	Hernstein
" 28-29	Ix 12189	2o Ny	1	Strelzhof 11,12
" 28-29	Ix 1219o	2o Ny	1	Strelzhof 13
" 28-29	Ix 12191	2o Ny	1	Strelzhof 13
" 28-29	Ix 12192	2o Ny	1	Strelzhof 13
" 28-29	Ix 12193	2o Ny	1	Strelzhof 13
" 28-29	1x 12212	2o Ny	1	Strelzhof 18
June 1-3	Ix 12233	5o Ny	1	St.Florian
" 1-3	Ix 12234	5o Ny	1	"
" 1-3	Ix 12235	5o Ny	1	"
" 1-3	Ix 12237	5o Ny	1	"
" 1-3	Ix 12246	50 Ny	1	"
" 1-3	Ix 12247	5o Ny	1	"
" 1-3	Ix 12248	5o Ny	1	"
" 1-3	Ix 12249	5o Ny	1	"
" 1-3	Ix 12251	5o Ny	1	"
" 1-3	Ix 12252	1o M	1	"
" 1-3	Ix 12253	1o M	1	"
" 1-3	Ix 12254	1o M	1	"
" 1-3	Ix 12255	1o M	1	"
" 1-3	Ix 12256	6 M	1	"
" 1-3	Ix 12257	8 M	1	"
Sept 2o-21	Ix 155o3 +)	1 F	1	"

+) see chapter EXPERIMENTAL LABORATORY INVESTIGATIONS (2)

Ny: nymphs
M : males
F : females

Table 3: Results of trapping excursions to Hernstein.

Excursion	Date	Total number of animals	Number of mice trapped	Among them First trapping	Among them Retrappings
	March 21-22	3	3	o	3
1	" 22-23	8	6	o	6
	" 23-24	9	9	1	8
	April 5-6	17	16	5	11
2	" 6-7	12	9	o	9
	" 7-8	7	6	1	5
	May 3-4	4	4	1	3
3	" 4-5	6	5	1	4
	" 5-6	4	4	2	2
	June 13-14	6	6	4	2
4	" 14-15	3	3	2	1
	" 15-16	11	1o	6	4
	July 25-26	8	8	5	3
5	" 26-27	15	11	7	4
	" 27-28	13	11	3	8
	Sept. 3-4	1o	1o	7	3
6	" 4-5	1o	9	2	7
	" 5-6	19	19	12	7
	Oct. 5-6	14	14	8	6
7	" 6-7	13	11	5	6
	" 7-8	13	11	8	3
	" 19-2o	2•	19	5	14
8	" 2o-21	9	8	6	2
		234	212	91	121

Table 4: Species of animals trapped.

Species	Number	Males	Females
Apodemus flavicollis	46	23	23
Clethrionomys glareolus	46	23	23
Microtus arvalis	13	6	7
Sorex araneus	14		
Sorex minutus	5		
Birds, div.spec.	3		
	127		

Table 5: Incidence of infestation of mice with ticks in Hernstein.

Month	Number of mice	Average per mouse of Larvae	Nymphs
March	19	1.o	-
April	31	3.7	o.1
May	11	12.2	o.1
June	14	15.6	o.4
July	25	6.4	o.1
August	0	-	-
Sept.	25	9,2	-
Oct.	29	7.8	-

Table 6: Results of mice trapping (Strelzhof).

Number of excursion	Date	Total number of trappings	Number of mice trapped first time	Number of retrappings
	March 25-26	1	1	-
1	" 26-27	-	-	-
	" 27-28	1	1	-
	May 5-6	1	-	1
2	" 6-7	2	2	-
	" 7-8	3	1	2
	May 28-29	2	2	-
3	" 29-3o	2	-	2
	" 3o-31	4	2	2
	Sept. 19-2o	7	6	1
4	" 2o-21	7	5	2
	Oct. 14-15	15	13	2
5	" 15-16	14	8	6
	" 16-17	11	6	5
	Nov. 12-13	22	14	8
6	" 13-14	19	18	1
		111	79	32

Table 7: Species of mice trapped in Strelzhof.

Apodemus flavicollis	59
Apodemus sylvaticus	6
Clethrionomys glareolus	8
Microtus arvalis	6
	79

Table 8: Incidence of infestation of Apodemus with ticks.

Month	Number of mice	Average of Larvae	Average of Nymphs	Maximum of Larvae	Maximum of Nymphs
March/April	2	1.3	0	3	0
May	4	65.0	1.3	137	2
May/June	6	87.6	0.5	145	2
September	9	22.1	0	52	0
October	26	9.4	0	31	0
November	19	1.4	0	8	0

Table 9: Serological investigation on sera of mice trapped in the Strelzhof area.

Species	Number of sera tested	NT-positive
Apodemus flavicollis	17	-
Apodemus sylvaticus	4	-
Clethrionomys glareolus	3	3
Microtus arvalis	1	-
Total	25	3

Table 10: Results of the NT with sera of small mammals and game collected in Hernstein.

Species	Number of sera tested	positive +)
Clethrionomys glareolus (Bank Vole)	159	6
Apodemus flavicollis (Yellownecked Mouse)	91	-
Microtus arvalis (Common Vole)	61	1
Sorex araneus (Common Shrew)	35	1
Apodemus sylvaticus (Field Mouse)	15	-
Pitymys subterraneus (Pine Vole)	10	-
Sorex minutus (Pigmy Shrew)	6	-
Neomys fodiens (Water Shrew)	2	-
Talpa europea (Mole)	1	-
Mus musculus (House Mouse)	1	-
Glis glis (Dormouse)	1	-
Capreolus capreolus (Roe Deer)	8	4
Cervus elaphus (Red Deer)	4	-
Rupicapra rupicapra (Chamois)	1	-
Lepus europaeus (Hare)	2	-
Vulpes vulpes (Fox)	4	2
	401	14

+) positive in a dilution 1:5

Table 11: Results of NT with sera of mammals from the Seewinkel area.

Species	Number of sera tested	positive
Lepus europaeus (Hare)	82	-
Capreolus capreolus (Roe Deer)	8	-
Vulpes vulpes (Fox)	6	-
Sus scrofa (Boar)	2	-
Erinaceus europaeus (Hedgehog)	39	-
Citellus citellus (Ground Squirrel)	85	-
Cricetus cricetus (Hamster)	13	-
Birds div.spec.	3	-
	238	-

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EXPERIMENTAL LABORATORY INVESTIGATIONS

(1) Identification of freshly isolated strains of TBE virus by means of the fluorescent antibody test (FAT).

In 1965, a study was started in order to investigate the use of the fluorescent antibody technique for the rapid identification of strains of TBE virus isolated in our field studies. It was stated in last year's report (1) that all 21 strains isolated in 1965 could be readily identified with this method in the first baby mouse passage.

In 1966, this technique was again applied whenever mice injected with ticks showed symptoms of disease indicating a possible virus isolation.

Smears were prepared from the brains of baby mice and stained with a fluorescein-labelled TBE conjugate as described in the previous report (1). For control purposes, conjugates against other arboviruses were used. In addition, the results of the FAT were controlled with the complement fixation (CF) test using crude extracts of infected mouse brains as antigens.

The results of the staining experiments are summarized in Table 1. From this it can be seen that a total of 26 virus strains could be identified with fluorescent antibodies. It must be emphasized that all tests were performed in the first mouse brain passage and that later the results could be confirmed with the CF test. In a few staining experiments, which are not incorporated in the table, the FAT was clearly negative. In these cases the suspected virus isolation proved to be actually a bacterial infection.

The results of our study show that the FAT is a valuable and reliable tool for the rapid identification of TBE virus.

Summary

Twenty-six virus strains isolated in the field studies could be rapidly identified with the fluorescent antibody technique in the first mouse brain passage.

Table 1: Identification of TBE virus with fluorescent antibodies.

Virus strain Ix.No	Isolated from	Days after infection of mice	Specific fluorescence	Control
12183	2o Ny	6	++++	0
12185	2o Ny	6	++++	0
12186	5 F	6	+++	0
12187	6 M	6	+++	0
12188	1 F	6	++	0
12189	2o Ny	7	++++	0
12190	2o Ny	7	++++	0
12191	2o Ny	7	++++	0
12192	2o Ny	7	++++	0
12193	2o Ny	7	++++	0
12212	2o Ny	6	++++	0
12233	5o Ny	5	++++	0
12234	5o Ny	5	++	0
12235	5o Ny	5	++++	0
12237	5o Ny	5	+++	0
12246	5o Ny	5	++++	0
12247	5o Ny	5	++++	0
12248	5o Ny	4	++++	0
12249	5o Ny	5	++++	0
12251	5o Ny	4	++++	0
12252	1o M	5	+++	0
12253	1o N	5	++++	0
12254	1o M	7	++++	0
12255	1o M	5	++++	0
12257	8 M	5	++++	0
15503	1 F	6	++++	0

Ny: nymphs
F: females
M: males

(2) Attempts at virus isolation by engorgement of ticks on baby mice

Russian authors recently claimed that males of Ixodes persulcatus, the principal vector of the subtype RSSE of TBE virus, probably play a role in the epidemiology of this virus (1). Due to the fact that males only suck for a short time, their bites are painless and, therefore, not noticed. This could, perhaps, account for those infections where the persons prior to disease had visited foci of TBE virus but do not give any history of tick bite.

In order to investigate whether or not males of Ixodes ricinus may transmit the virus by bite, an excursion was carried out in the fall to St.Florian near Linz. In this area a considerably high number of virus-contaminated male ticks had been found in the spring (see: Field studies).

Materials and Methods: Starving and active nymphs and adults of Ixodes ricinus were collected in the usual manner in a mixed pinewood forestation near St.Florian on September 20-21. In the laboratory, about half the number of nymphs were homogenized in pools of five individuals. They were suspended in PBS containing 10 % horse serum and inoculated i.c. into baby mice. The rest of the nymphs and all adults were made to suck on baby mice. For this purpose, a baby mouse and a tick were transferred into a glass tube, which was closed by a wire gauze. The ticks, together with mice, were incubated at + 30° C for five to six hours. At the end of this time, practically all ticks with the exception of male adults were sucking. Then the baby mice were brought back to their mothers. The animals were observed for 3 weeks.

Results: A total of 133 nymphs was tested in 26 pools by the conventional method. No virus could be isolated. However, from 187 nymphs and 13 females, which had engorged on the mice, one strain of virus was isolated from a mouse which became infected by a female tick. From 19 males which had been tested in this manner, no virus could be isolated.

This method of virus isolation is very cheap and can be easily and rapidly done. As regards the main aim of this study, the number of males tested was too low to draw any definite conclusions. Further studies will be done on this subject.

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(3) Breeding experiments on the innate resistance of Mus musculus spicilegus to TBE virus

In 1963, innate resistance to TBE could be demonstrated in a line of Mus musculus spicilegus from Laxenburg, south of Vienna (1). From this line, some males were available for further experiments.

Material and Methods:

M i c e : Mus musculus spicilegus males from Laxenburg, a line bred in the laboratory (third generation) originating from two pairs, caught in the neighbourhood of Schloss Laxenburg.

Mus musculus spicilegus females caught in the cellar of the Museum of Natural History in Vienna.

An inbred strain of white laboratory mice (Institute of Hygiene, Vienna) with high susceptibility to TBE.

V i r u s : TBE virus, subtype CEE, strain Will.III which was isolated from ticks caught in the "Strelzhof" focus near Willendorf.

The mus musculus spicilegus males from the Laxenburg strain and the females from the Museum were mated and the third generation of this crossing was mated with susceptible laboratory mice; with F 1 and F 2 generations of these hybrids resistance to TBE was tested by inoculating s.c. o.2 ml of a 1o % suspension of infected baby mouse brains. The F 1 generation was divided into 7 small groups, and these were inoculated with a dilution series from $1o^{-2}$ to $1o^{-8}$ (o.3 - 3 mill. LD_{5o}). The F 2 generation was divided into two groups which were inoculated with the dilutions $1o^{-5}$ (1oo LD_{5o}) and $1o^{-7}$ (1 LD_{5o}). For control purposes, the virus was also titrated in white mice.

Results: From the F 1 generation 8 of the 15 mice died. At all concentrations higher than $1o^{-8}$, mice died and survived in an irregular manner (Table 1). The average survival time was 8.9 days for the laboratory mice and 11.8 days for the F 1-hybrids. From the F 2 generation, 44 specimens were inoculated with 1oo LD_{50}. From this group, 8 animals survived and 36 died between the 7th and 18th day (average survival time: 9.7 days). Forty-six specimens were inoculated with 1 LD_{50}. Twenty-one of them died between the 7th and 17th day (average survival time: 1o.5 days). In the control group, the average survival time was 7 days at $1o^{-5}$, 9 days at $1o^{-7}$ (Table 2).

Excluding the 2 mice inoculated with only o.3 LD_{50} (dilution $1o^{-8}$), there is a 62 per cent mortality in the F 1 generation. The F 2 generation shows a mortality of 82 % in the group inoculated with a sufficient quantity of virus. The difference between the mortality rate of the F 1 and the F 2 generation indicates that the P generation, a line of Mus musculus spicilegus deriving from Laxenburg and females from the Center of Vienna, was not homocygote for resistance to TBE. The resistance is probably based on a single dominant gene, but further experiments are necessary to prove this assumption. One can only speculate on the reason of the susceptibility to TBE in strains of Mus musculus spicilegus. This may be due to accidental hybridization with white mice which escaped from laboratories. Another possibility is that the mice lost that specific gene as a result of domestication. The fact that the average survival time of laboratory mice and the F 1 and F 2 generation hybrids differed may be explained by an age difference between the groups. However, it is conceivable that other innate factors account for this discrepancy.

The next task is to investigate a new line of Mus musculus spicilegus with regard to its homocygosity and to breed a homocygote resistant line. Another task is to compare this innate resistance with the resistance gene in the C3HRV strain, developed by Göschel and Koprowsky (2).

Summary: The nature of an innate resistance to TBE virus previously found in strains of Mus musculus spicilegus was investigated. For this purpose, these strains were mated with white mice which invariably succumb to the infection. The results indicate that the resistance is probably associated with a single dominant gene, but more work has to be done to confirm this result.

Table 1: Comparison of the sensitivity of F 1 mice and laboratory mice to TBE virus (Number of mice dead/total number of mice).

		Days after infection	Virus dilution 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Death rate of mice	F 1 Concentration	6	0/2	0/1	0/2	0/3	0/3	0/2	0/2
		7	0/2	0/1	0/2	0/3	0/3	0/2	0/2
		8	0/2	0/1	0/2	0/3	0/3	0/2	0/2
		9	0/2	0/1	0/2	0/3	0/3	0/2	0/2
		1o	0/2	0/1	0/2	0/3	0/3	0/2	0/2
		11	1/2	0/1	2/2	1/3	1/3	0/2	0/2
		12	1/2	0/1	2/2	3/3	1/3	0/2	0/2
		13	1/2	0/1	2/2	3/3	1/3	0/2	0/2
		14	1/2	0/1	2/2	3/3	1/3	0/2	0/2
		15	1/2	0/1	2/2	3/3	1/3	1/2	0/2
		16	1/2	0/1	2/2	3/3	1/3	1/2	0/2
		17	1/2	0/1	2/2	3/3	1/3	1/2	0/2
		18	1/2	0/1	2/2	3/3	1/3	1/2	0/2
		19	1/2	0/1	2/2	3/3	1/3	1/2	0/2
		2o	1/2	0/1	2/2	3/3	1/3	1/2	0/2
		21	1/2	0/1	2/2	3/3	1/3	1/2	0/2
		22	1/2	0/1	2/2	3/3	1/3	1/2	0/2
of mice	Control	6	1/5	0/5	0/5	0/5	0/5	0/5	0/5
		7	2/5	4/5	1/5	4/5	2/5	0/5	0/5
		8	4/5	4/5	4/5	4/5	3/5	2/5	1/5
		9	5/5	5/5	5/5	5/5	5/5	15/5	1/5
		1o	5/5	5/5	5/5	5/5	5/5	5/5	1/5
		11	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		12	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		13	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		14	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		15	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		16	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		17	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		18	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		19	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		2o	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		21	5/5	5/5	5/5	5/5	5/5	5/5	2/5
		22	5/5	5/5	5/5	5/5	5/5	5/5	2/5

Table 2: Comparison of the sensitivity of F 2 mice and laboratory mice to TBE virus (Number of mice dead/total number of mice).

		Days after infection	Virus dilution 10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Death rate of mice	F 2 Generation	6		0/44		0/46	
		7		4/44		1/46	
		8		15/44		3/46	
		9		22/44		7/46	
		10		25/44		11/46	
		11		30/44		18/46	
		12		33/44		18/46	
		13		33/44		18/46	
		14		34/44		18/46	
		15		34/44		19/46	
		16		34/44		19/46	
		17		34/44		21/46	
		18		36/44		21/46	
		19		36/44		21/46	
		20		36/44		21/46	
		21		36/44		21/46	
		22		36/44		21/46	
	Control	6	0/4	0/4	0/4	0/4	0/4
		7	4/4	4/4	1/4	0/4	0/4
		8	4/4	4/4	2/4	0/4	0/4
		9	4/4	4/4	2/4	2/4	0/4
		10	4/4	4/4	3/4	2/4	0/4
		11	4/4	4/4	3/4	2/4	0/4
		12	4/4	4/4	3/4	2/4	0/4
		13	4/4	4/4	3/4	2/4	0/4
		14	4/4	4/4	3/4	2/4	0/4
		15	4/4	4/4	3/4	2/4	0/4
		16	4/4	4/4	3/4	2/4	0/4
		17	4/4	4/4	3/4	2/4	0/4
		18	4/4	4/4	3/4	2/4	0/4
		19	4/4	4/4	3/4	2/4	0/4
		20	4/4	4/4	3/4	2/4	0/4
		21	4/4	4/4	3/4	2/4	0/4
		22	4/4	4/4	3/4	2/4	0/4

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(4) Studies on receptor substances for TBE virus

(a) Chemical investigations concerning competitive inhibitors of hemagglutination by TBE virus.

Our experiments with strain VIE 415 B of TBE virus, subtype CEE, led to a method which made it possible to measure the competitive inhibition of hemagglutination (HA) at the pH optimum of this reaction. The method was extensively described in the report for 1964 (1). We obtained lipid extracts from mouse brain by homogenizing them with the 20-fold amount of chloroform-methanol 2:1 according to Folch et al (2) and dialyzing the extract as proposed by Folch et al (3). When the hemagglutination-inhibiting (HI) activity of homogenates from mouse brain and the lipid extracts therefrom were tested with this new method, they were 100 times more active (4), than when tested by older methods (5).

We have now extended these findings to homogenates and lipid extracts from brains of other mammals. No significant differences in the HI capacity of the different extracts could be observed, but in all cases the HI activity at pH 6.4 was about 100 times higher than at pH 9 (Table 1). Also, extracts from other organs showed no essential differences in their HI capacities, which were comparable to that of brain extract (Table 2). The HA of a protamine sulfate treated preparation of West Nile virus, which belongs to the serological group B of arboviruses, as does also TBE virus, was equally sensitive to lipid extracts from mouse brain, whereas the HA of Sindbis virus, belonging to group A, was not inhibited by our extracts.

It has already been mentioned in the previous report (4) that the HI activity was exhibited by the more polar lipids, which could be separated from the inactive neutral fats, cholesterol and cholesterol esters by precipitation from chloroform with the 6-fold amount of acetone.

The phosphatides and cerebrosides so obtained, which were able to inhibit the HA of the TBE virus, could be further fractionated by column chromatography on silica gel (4). Three fractions were obtained.

The composition of these fractions was investigated with a two-dimensioned method of thin layer chromatography, which we developed from the original method of Abramson and Blecher (6). We are now able to identify the position of the following substances on the chromatogram: Phosphatidyl-inositol-diphosphate, gangliosides, lysophosphatidyl-cholin, phosphatidyl-cholin, phosphatidyl-serine, phosphatidyl-inositol, phosphatidyl-cholamin, cerebroside-sulfuric esters, kerasin, phrenosin, phosphatidic acids, cholesterol, cholesterol esters and impurities. From these, the position of phosphatidyl-serine was erroneously assumed to be nearly identical with the position of phosphatidyl-cholamin (4). This has to be corrected in the sense that the position of phosphatidyl-serine is near the position of phosphatidyl-inositol. As a consequence of this, the composition of the fractions from the column-chromatography is as follows: I contained phosphatidic acids, cerebrosides (phrenosin and kerasin) and cerebroside sulfuric esters, II consisted of phosphatidyl-cholamin and III contained phosphatidyl-serine, phosphatidyl-inositides, phosphatidyl-choline (lecithine) and sphingomyeline. Only this last fraction showed the HI activity.

By a second chromatographic step on a column of silica gel with a slightly different activity, the group of substances showing HI inhibition could be further fractionated. Again, three fractions were obtained. With three parts chloroform and two parts methanol, phosphatidyl-serine together with the phosphatidyl-inositides was eluted and the same solvents in relation 1:1 brought down phosphatidyl-choline and sphingomyeline in two well separated peaks. The whole procedure of separations is shown schematically in Fig.1.

The HA inhibition test at pH 6.4 indicated that none of the fractions of the second step was active. However, a mixture of all three showed again the full capacity to inhibit the HA of TBE virus. Therefore, combinations of all chromatographically obtained fractions were tested in regard to their ability to inhibit the HA of the virus at pH 6.4. The result is sum-

marized in Table 3. Only such pairs showed a strong HI activity, in which fraction IIa was combined with lecithin or with sphingomyelin. But the function of lecithin or sphingomyelin seems to be only an auxiliary one. These two phosphatides could be replaced by cetyl-trimethyl-ammonium bromide (Cetavlon), a substance which does not occur naturally (Table 4).

As yet, it cannot be stated, which of the components of fraction IIIa is the biologically active one. Phosphatidyl-serine was not available to us in a form giving only a single spot on the two-dimensional chromatogram. Our preparation did not contain phosphatidyl-inositol, but contained very probably phosphatidyl-inositol diphosphate. It showed HI activity together with lecithin, but not alone. Recently, phosphatidyl-inositol and phosphatidyl-inositol-diphosphate could be obtained in a chromatographically pure state from a commercial source (Koch-Light Lab.). According to a personal letter from A.P.Joshi, the two products were prepared from ox brain following the procedure from Folch (7) and further purified following the methods of Long and Owens (8) and Hendrickson and Ballou (9) respectively. Phosphatidyl-inositol was not active, but phosphatidyl-inositol-diphosphate was able to inhibit the HA of the virus when combined with lecithin.

It is far too early to draw final conclusions, but the available information points to the assumption, that the HI substance is most probably phosphatidyl-inositol-diphosphate in combination with certain trimethyl-ammonium compounds. However, it is also possible that the HI activity has to be ascribed to an as yet unknown substance belonging to the group of lipid haptenes, which are also activated by an auxiliary lipid (10).

In this connection it must be mentioned, that phosphatidyl-inositol-diphosphate can be degraded by a specific enzyme only when combined with certain trimethyl-ammonium compounds (11). The same authors could demonstrate that the effect of the enzyme-activating amphipathic substances is due to their ability to form micelles with the phosphatidyl-inositol-diphosphate. On

the surface of these micelles the substrate molecules are arranged in parallel alignment and this seems to be a necessary condition for the proper attachment of the specific enzyme.

We think that our preliminary results suggest a rather similar mechanism for the adsorption of TBE virus onto the surface of the red cell. It is generally accepted that cell membranes contain a double layer of parallel-arranged lipid molecules, their polar head showing outwards and their monopolar tails pointing towards the core of the membrane (12). It seems that TBE virus is able to attach itself to certain lipid receptor molecules, which occur in this double layer in a well ordered arrangement. However, the same or similar substances in solution can only then compete with the receptors in the cell membrane, when they are brought into a similar arrangement by the micelle-forming action of certain trimethyl-ammonium compounds.

Summary: Lipid substances, extractable from brain and other organs, exhibit a strong hemagglutination-inhibiting activity for TBE virus, when tested under competitive conditions at pH 6.4. Fractionation by column-chromatography on silica gel showed that no single lipid alone was able to inhibit the hemagglutination (HA) of the virus. By testing different combinations, it was found that for the full action of the HA inhibition, the presence of certain auxiliary lipids, containing a trimethyl-ammonium group, was necessary. The receptor analogue inhibitor was very probably phosphatidyl-inositol-diphosphate, but the presence of some other substance of lipid haptene character could not be excluded. The possible mechanism of hemagglutination inhibition is discussed.

Table 1: HA-inhibiting activity of homogenates and lipid extracts of brains from different mammals.

Material	pH 9.0	pH 6.4
Homogenates:		
Laboratory mouse	1 : 32o	1 : 1o2,4oo
Field mouse	1 : 64o	1 : 2o4,8oo
Guinea pig	1 : 2,56o	1 : 256,ooo
Ox	1 : 64o	1 : 51,2oo
Cat	1 : 2,56o	1 : 256,ooo
Monkey	1 : 5,12o	1 : 256,ooo
Extracts:		
Laboratory mouse	6o µg/o.8 ml	o.3o µg/o.8 ml
Field mouse	6o µg/o.8 ml	1.25 µg/o.8 ml
Guinea pig	1o µg/o.8 ml	o.o7 µg/o.8 ml
Ox	5o µg/o.8 ml	o.6o µg/o.8 ml
Cat	2o µg/o.8 ml	o.6o µg/o.8 ml
Monkey	2o µg o.8 ml	o.6o µg/o.8 ml

Table 2: HA inhibiting activity of lipid extracts of different origin.

Brain (Rat)	0.15 µg/0.8 ml
Liver (Rat)	0.07 µg/0.8 ml
Kidney (Rat)	0.30 µg/0.8 ml
Lung (Rat)	0.07 µg/0.8 ml
Muscle (Rat)	0.15 µg/0.8 ml
Erythrocytes (Goose)	0.30 µg/0.8 ml
Erythrocytes (Man)	0.15 µg/0.8 ml

Table 3: HA inhibiting capacity of chromatographically separated lipids and of combinations therefrom.

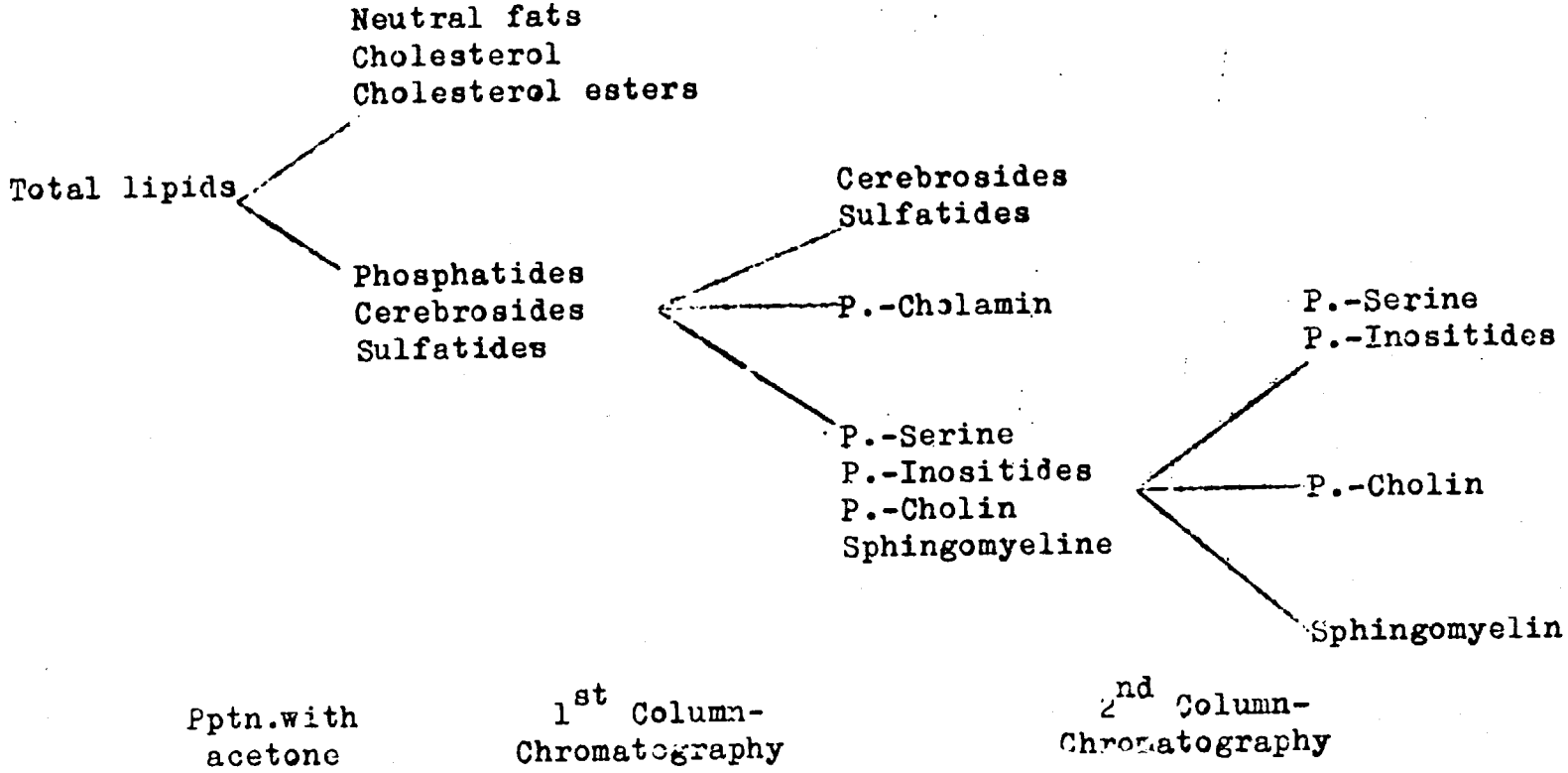
	1st Exp.	2nd Exp.
Fractionation I (Mouse brain phosphatides)		
I a (Cerebrosides + Sulfatides)	-	-
I b (P.-Cholamin)	-	-
I c (P.-Serine, Inositides P.-Cholin, Sphingomyelin)	+ +	+ +
I (a + b)	n.d.	-
Fractionation II (Substances of I c)		
II a (P.-Serine + Inositides)	-	-
II b (P.-Cholin)	-	-
II c (Sphingomyelin)	-	-
II (a + b + c)	+ +	+ +
II (a + b)	+ +	+
II (a + c)	+	+
II (b + c)	-	-
Ia + IIa	±	±
Ia + IIb	±	±
Ia + IIc	-	-
Ib + IIa	-	-
Ib + IIb	-	-
Ib + IIc	-	-

++ very strong activity
+ strong activity
± weak activity
- no activity
n.d. not done

Table 4: HA inhibiting activity of the substances of peak IIa from column chromatograms when combined with some trimethylammonium compounds.

Smallest amount of combinations still active at pH 6.4		
Fraction IIa + Lecithin	Fraction IIa + Sphingomyelin	Fraction IIa + Cetavlon
0.08 µg/0.8 ml	0.08 µg/0.8 ml	0.02 µg/0.8 ml
0.04 µg/0.8 ml	0.04 µg/0.8 ml	0.04 µg/0.8 ml
0.08 µg/0.8 ml	0.15 µg/0.8 ml	0.08 µg/0.8 ml
0.15 µg/0.8 ml		
0.04 µg/0.8 ml		

Fig.1: Scheme of separation of lipids extracted from mouse brain.



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b) Investigations concerning competitive inhibition of the adsorption of TBE virus onto cells.

In a series of studies, the question of whether or not the HI substance can also inhibit the infectivity of the virus was investigated. This was done with the assumption that the lipids can only be called receptors for the virus if th · are able to compete with cells in which the virus can propagated.

At first this question was studied with TBE virus, employing the plaque technique in chick embryo cells. However, under agar overlay, using the method recommended by Porterfield (1), if any plaques at all were obtained, they could only be read with great difficulty. The addition of DEAE dextran to the agar (2) did not improve the plaque-forming properties of the virus.

Later it was found that the HA of West Nile virus, which belongs to the group B of arboviruses as does TBE virus, can also be inhibited by lipid extracts from mouse brains (see page 26). Since West Nile virus gives clear-cut plaques in chick embryo cells and is not hazardous to work with, this virus was chosen as a model in further experiments. The inhibiting effect of the lipids was also assayed in an established kidney cell line of African green monkeys by means of the combined application of the fluorescent antibody technique (FAT) and of microphotometry.

I. Studies with fluorescent antibodies:

Materials and methods:

Virus: The 13th mouse brain passage of strain "Egypt 101" was used. Brains of infected baby mice were homogenized in a 0.15 molar phosphate buffer at pH 7.6 containing 10 % horse serum. After centrifugation at 10,000 r.p.m. for 1 hour, the stock virus was frozen in 1 ml portions at -80^{o}C.

Tissue culture methods: An established kidney cell line of African green monkeys was grown in 160 ml French square bottles in a growth-promoting medium consisting of 80 % TCM 199 and 20 % calf serum supplemented with antibiotics (100 units of penicillin, 100 mg of streptomycin).

For the tests, the cells were propagated in Leighton tubes in which a coverglass measuring 11 x 32 mm was placed. Prior to viral inoculation, the growth-promoting medium was replaced by a maintenance medium consisting of 95 ml Eagle's minimum essential medium (with Hanks' salts) and 5 % calf serum.

Lipid extracts: Lipid extracts from mouse brains or brains of guinea pigs were prepared as described above (see page 26). The lipids were emulsified in phosphate buffer pH 6.4 at a concentration of 0.1 mg lipid/ml buffer.

Performance of test: Equal parts of purified virus and lipid extract were mixed and kept for 15 minutes in an ice bath. To the virus control an equal amount of phosphate buffer without lipids was added. Then 0.1 ml of the virus-lipid mixture or of the virus control was added to the tissue culture tubes. After 60 minutes, the fluid was replaced by new maintenance medium. Changes of medium were made at daily intervals.

After one, two and three days, coverglasses were removed and stained for 20 minutes with a fluorescein-labelled West Nile antiserum according to methods previously described (3).

Fluorescence microscopy and microphotometry: The Reichert Zetopan microscope equipped with a Binolux fluorescence outfit was used. A darkfield condenser was employed in combination with a UG 1 (1.5 mm) exciter filter and a GG 13-Wratten 2 B barrier filter.

Microphotometry was done with the Reichert microphotometer, which was set to give lowest sensitivity. The largest measuring diaphragm in combination with a 5 x ocular and a 20:1 objective was used. Thus the virus specific fluorescence of several cells was registered with one measurement.

In order to compensate for the nonspecific fluorescence of the cells, the fluorescence of noninfected cells on the coverglass was measured first and the needle of the registering instrument then readjusted to its zero point. This was repeated several times during the measuring process.

Results:

One day after infection, only a few cells exhibited specific fluorescence, thus making it possible to count all infected cells on a coverglass. As can be seen in Table 1, the 2 coverglasses on which the effect of the lipids was assayed contained only 1/10 or less of infected cells as compared with the controls.

On the second day, the infected cells were too numerous to be counted. It was also not possible to count the numbers of infected centers in the cell monolayers. Microphotometry was, therefore, performed measuring 100 adjoining fields on each coverglass. In three different experiments the brightness of the fluorescence was measured in cells of 15 coverglasses (7 in which the lipids were tested and 8 controls). Upon comparison of the figures given in the table, it will be noted that the lipids clearly had an inhibitory effect on the virus. The intensity of the fluorescence obtained with the controls was at least 4 times but mostly more than 100 times higher than those measured in the cells infected with the lipid-treated virus. By adding up all figures and taking the mean therefrom, one arrives at a difference in the intensity of the fluorescence of 10:1.

On the third day, specific fluorescence of the controls was still brighter but the difference was no longer marked. This was certainly due to infection of cells with newly formed virus.

II. Studies with the plaque technique:

Materials and methods:

Tissue culture methods: Tissue cultures were prepared in 160 ml French square bottles from 9 day old chick embryos. The growth-promoting medium consisted of 97 % TCM 169 and 3 % calf serum to which several antibodies were added.

Assay of virus inhibition: Essentially the same technique was used as described under I. After incubation in an ice bath, ten-fold dilutions were made of the virus-lipid mixtures. One ml of a virus dilution was added to each tissue culture bottle from which the medium

had been removed. The bottles were kept for 60 minutes at 37°C. Thereafter, the 1 ml was pipetted off and an agar overlay was introduced consisting of the Tris-Gey medium as recommended by Porterfield (1). However, neutral red was omitted. Two days later, a second overlay with neutral red was added. The plaques were read on the third day.

Results:

On the third day, plaques were present with a diameter of 3-4 mm. Also with this method a similar inhibitory effect of the lipids on the virus was noticeable, as was observed with the fluorescent antibody technique. The results of two typical experiments recorded in Table 2 show that about 10 times more plaque-forming units were obtained with the virus controls than with the lipid-treated virus.

Conclusions and recommendations:

From the results obtained with both methods, it appears that lipid extracts from brains of mice and guinea pigs can not only compete with the hemagglutination but also with the infectivity of West Nile virus. Because of the experimental conditions used, it is evident that the lipids prevented the viria from adsorption onto the cells. However, it is striking that approximately only 90 % of the virus particles were inhibited.

We can, therefore, only tentatively conclude that the lipids act as a receptor for the virus. To confirm this, further studies have to be done along this line. Experimental conditions will be changed with the aim of preventing infection completely. In addition, the effect of the lipids will also be tested on TBE virus employing fluorescent antibodies. Perhaps by replacing the agar with other compounds, it will be possible to develop an overlay medium for the plaque technique which is also suitable for TBE virus.

Summary:

Lipid extracts from brains of mice and guinea pigs were tested for their ability to inhibit competitively the infection of tissue culture cells with West Nile virus. This virus was used as a model in place of TBE virus. The virus was propagated in chick embryo and African green monkey cells and the assay of virus multiplication was made with the plaque technique and by the combined application of the fluorescent antibody method and microphotometry. The preliminary results indicate that the lipids which can inhibit the adsorption of virus onto erythrocytes can also prevent its adsorption onto tissue culture cells.

Table 1: Effect of lipids on West Nile virus assayed by the combined application of the fluorescent antibody technique and microphotometry.

		Days after infection		
		1	2	3
Experiment number	Virus	Number of fluoresc. cells	Intensity of fluorescence	Intensity of fluorescence
1	Li	16	86+), 47, 439	1673, 1724
	Co	172	3951, 3090, 1969	4209
2	Li	4	811, 623	
	Co	207	3477, 3084, 2618	
3	Li		81, 62	4123
	Co		1220, 3300	7179

Li: Treated with lipids
Co: Control
+): Each figure is the sum of measurements done on a cover-glass in 100 adjoining fields and represents points on the scale of the registering instrument.

Table 2: Effect of lipids on virus assayed by the plaque technique.

Dilution	Number of plaque-forming units			
	Experiment 1		Experiment 2	
	Lipid	Control	Lipid	Control
10^{-3}	62	100	10	100
10^{-4}	5	42	2	11
10^{-5}	0	6	0	2
10^{-6}	0	0	0	0

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(5) The physical properties of complement-fixing antibodies following infection with TBE virus

Our previous studies of the immunologic response of man to TBE virus (1) have shown that neutralizing antibodies consist of 19 S - macroglobulins in the early stage and of 7 S γ_2-globulins in the late stage of the disease. By contrast, complement-fixing (CF) antibodies were invariably associated with 7 S γ_2-globulins. The antigens used for the CF test were ether-acetone-extracts prepared from infected brains of adult mice. With these antigens, CF antibodies were first detectable in the third week of disease. Later, Kunz and Krausler (2) found that CF antibodies can be consistently demonstrated during the first days of TBE if sucrose-acetone preparations of infected brains of baby mice are used for the test.

This present study was done in order to determine the nature of these early CF antibodies.

Materials and Method:

Antigen: The 11th baby mouse passage of the Austrian TBE strain "Vie 415 B" was used. The antigen was prepared from baby mouse brain with the sucrose-acetone method of Clark and Casals (3).

Sera: From each TBE patient two sera with a minimum titer of 1:32 were investigated. The first serum was drawn on the day of hospitalization and the second was taken during convalescence. The days of disease given in Table 1 were calculated from the day when the first symptoms of the disease of the central nervous system (i.e., the second phase of TBE) appeared.

(I). In a first series of tests, sera from 10 patients were tested separately. The sera were separated by means of ultracentrifugation in a NaCl density gradient according to the method of Kunkel et al. (4) as modified by Franklin (5). Layers of 21 %, 14 % and 7 % saline were introduced into a cellulose nitrate tube. After the serum was placed upon the gradient, centrifuga-

tion was performed in a Spinco centrifuge using the SW 25 rotor at 21,500 rpm until a total of 18 mill. revolutions was reached.

(II). In a second series of tests 2 separate pools of 5 early and 5 late sera were separated by column chromatography using Sephadex G - 200 in 0.1 ml Trisbuffer (pH 8.0). After dialysis against buffered saline (0.15 m, pH 7.0), the fractions were tested in the CF.

(III). The protein concentration of the fractions was determined with the Biuret reaction. The nature of the proteins in the fractions was determined by electrophoresis. The complement-fixation (CF) tests were done with a drop-type technique described previously (2).

Results:

(a) Single sera (Table 1):

Upon comparison of the fractions containing mainly 19 S globulins with those containing mainly 7 S globulins, it can be seen that in 9 out of the 10 "early" sera, the maximum CF activity was present in the 7 S range. Only in one case (V 275/66) were almost identical CF titers found in the 7 S and 19 S fraction. In the "late" sera, the antibodies were solely of the 7 S variety. Due to diluting of the sera through the necessary manipulations, utilizable results were consistently obtained with sera which had a CF titer of at least 1:64.

In sera with minimal titers of 1:8 in the 7 S fraction, a weakly positive CF was also obtained occasionally in the 19 S region (V 630/66, 1st serum; V 272/66, 2nd serum; V 158/65, 2nd serum). However, immunoelectrophoresis revealed the presence of 7 S γ_2 globulins in these 19 S fractions.

(b) Pooled sera:

In these tests two serum pools were used (serum A and serum B). Pool A consisted of sera which had been collected during the first week of the disease. Sera collected from the 4th to 6th weeks were combined in pool B. The three peaks of the protein concentration are listed in Table 2 as fraction I, II and III.

With both pools, the bulk of the activity was found in fraction II. Analysis by electrophoresis showed that this fraction contained 7 S γ globulins and albumins, but no 19 S - macroglobulins. A small amount of activity was also found in fraction I, which consisted of 19 S and of 7 S globulins. Fraction III, where albumins were predominately located, was negative in the CF test.

Discussion: Fractionation of sera using either density gradient-ultracentrifugation or column chromatography yielded similar results. Maximal CF antibody titers were obtained in the fraction which was devoid of 19 S -macroglobulins and contained mainly 7 S γ globulins. The smaller amount of CF activity which was found in the fraction where the 19 S globulins resided was obviously due to contaminations with 7 S globulins. Only in one case ("early"serum of patient V 275/66) were equal titers found in the 7 S and in the 19 S region, so that the presence of 19 S type antibodies could not be excluded.

The results of our study concur with similar investigations by other authors. Bellanti et al. (6) observed, after experimental infection of guinea pigs with Japanese encephalitis and TBE (subtype RSSE) viruses, only CF antibodies of the 7 S type.

These findings are of interest because the primary response to immunization usually leads to the formation of 19 S antibodies which are later followed by the 7 S variety. This is also true for the production of neutralizing antibodies after infection with poliomyelitis (7), TBE (1) and Japanese encephalitis (6) viruses, where the first detectable neutralizing activity is of the 19 S and later of the 7 S γ globulin type.

At present, nothing is known about the cause of this discrepancy between the CF and the neutralizing antibodies. One wonders, therefore, if it could not be explained by the presence of different types of antigens. In connection with this, it is of interest that a primary response of 7 S antibodies was found after the application of some soluble antigens (8,9). We cannot answer

at present the question of whether or not in our case the formation of CF antibodies was due to a soluble viral antigen. So far, the occurrence of a soluble antigen was suggested only with Japanese B encephalitis virus (10) which is like TBE virus, an arbo-group B agent. Perhaps the findings of Mussgay and Horzinek (11) also point in the same direction. After treating Sindbis virus with cobra venom, these authors obtained non-hemagglutinating non-infectious but complement-fixing components which they interpreted as being the core of the virus.

Summary: Sera drawn in the acute stage of disease and during convalescence from TBE patients were fractionated by either column-chromatography or by ultracentrifugation in a density gradient. Complement-fixing antibodies were predominantly found in the fraction where the 7 S γ globulins resided in both the early and the late sera. The reason for this primary 7 S immune response is discussed.

Table 1: Titers in the CF separated into 3 fractions by density gradient-ultracentrifugation.

Serum No.		Day of disease	19 S-Fract.	Intermed. Fract.	7 S-Fract.
V 630/66	1st s.	3	1:2	-	1:8
	2nd s.	22	neg.	1:8	1:16
V 272/66	1st s.	3	neg.	neg.	neg.
	2nd s.	26	1:2	neg.	1:8
V 366/66	1st s.	3	neg.	1:2	1:2
	2nd s.	26	neg.	1:4	1:8
V 242/66	1st s.	5	neg.	1:2	1:2
	2nd s.	32	neg.	1:4	1:8
V 275/66	1st s.	6	1:2	1:2	1:2
	2nd s.	20	neg.	1:8	1:8
V 504/66	1st s.	5	neg.	1:2	1:4
	2nd s.	26	neg.	1:2	1:8
V 696/66	1st s.	7	neg.	1:4	1:8
	2nd s.	17	neg.	1:4	1:8
V 554/66	1st s	7	neg.	1:4	1:4
	2nd s.	40	neg.	1:4	1:4
V 158/65	1st s.	5	neg.	1:2	1:4
	2nd s.	27	1:2	1:4	1:8
V 478/65	1st s.	7	neg.	u.	1:4
	2nd s.	21	neg.	u.	1:8

u: undiluted serum positive

Table 2: Titers in the CF and type of protein in fractions of pooled sera separated by Sephadex-chromatography.

Serum pool	CF titer	Week of disease	Fraction I: CF titer	Fraction I: Protein Alb.	Fraction I: α_1	Fraction I: M	Fraction I: α_2	Fraction I: γ_1	Fraction I: A	Fraction II: CF titer	Fraction II: Protein Alb.	Fraction II: α_1	Fraction II: M	Fraction II: α_2	Fraction II: γ_1	Fraction II: A	Fraction III: CF titer	Fraction III: Protein Alb.	Fraction III: α_1	Fraction III: M	Fraction III: α_2	Fraction III: γ_1	Fraction III: A
A	1:128	1	u	-	+	±	+			1:4	±	-	+	±			neg.	+	-	-	-		
B	1:512	4-6	1:2	-	+	±	-			1:8	±	-	+	-			neg.	+	-	-	-		

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(6) Persistence of complement-fixing and hemagglutination-inhibiting antibodies after overt TBE

In a previous study carried out on a small number of former TBE patients of the hospital in Neunkirchen (Lower Austria), complement-fixing (CF) antibodies could still be detected in their sera 6 years after disease (1). This was surprising since it is generally accepted that this type of antibody tends to disappear after virus infections more rapidly than hemagglutination-inhibiting (HI) and neutralizing antibodies.

In order to extend our knowledge further, former patients of this hospital who had contracted TBE between 1958 and 1961 were recalled in 1967 for rebleeding. The sera were tested in the CF and the HI test. Whenever possible the convalescent sera which had been kept in a deep freezer were also investigated.

Materials and methods:

Forty-two people were included in this study. Two of them were ill in 1958, seven in 1959, sixteen in 1960 and seventeen in 1961. The CF was performed as previously described (1). The HI test was done with 4-8 units of antigen according to the methods of Clarke and Casals (2). In both tests the same antigen was used which was prepared by sucrose-acetone treatment of brains from baby mice.

Results:

Due to technical reasons only 39 sera could be evaluated in the CF test. All but one serum still contained CF antibodies 6-9 years after the disease. As can be seen in Table 1, the titers ranged from 1:4 - 1:64, with the majority at 1:8 - 1:16. Convalescent sera from 34 of these persons and sera from 32 persons taken the year after the disease were still available. It can be noted that the convalescent sera had a very high titer (1:32 - 1:512), most of them being positive at dilutions of 1:64 - 1:128. By contrast, the titers of the sera drawn 1 year after disease (Table 3) showed no essential

difference when compared with those which were recorded 5-7 years thereafter. In only 5 cases did the titers drop 2 steps during this period.

From this it is evident that CF antibodies show a definite change in titer during the year following disease but then tend to remain at the same level over an unknown number of years.

All of the 42 persons tested in the HI still had antibodies in their serum 6-9 years after TBE. Titers ranged from 1:8-1:1280 (Table 4). The bulk of the sera was positive at dilutions of 1:80 or 1:160.

From 39 of the 42 persons convalescent sera were tested giving titers of 1:80 - 1:10,240 with most sera being in the 1:320 - 1:1280 range. Thus also the HI antibodies show a change in titer in the months following disease.

In Table 5 examples of CF and HI antibodies of 15 patients are recorded. Most sera show a ratio between CF and HI antibodies of 1:5 - 1:20 but there are exceptions to this rule. In the table it can further be seen that the level of the antibodies reached during disease does not permit a conclusion to be drawn as to which titer will be retained later.

Summary:

Forty-two persons who had contracted TBE 6-9 years previously were rebled in 1967 and tested for the persistence of complement-fixing (CF) and hemagglutination-inhibiting (HI) antibodies.

From 39 persons tested 38 still had CF antibodies. As a rule a drop in antibody titer could be demonstrated one year after the disease, but then the antibodies tended to be retained at the same level for the period under investigation.

In all the sera of 42 persons tested in the HI antibodies were still detectable. With few exceptions high titers in the CF test concurred with high titers in the HI-test.

Table 1: CF antibody of 39 persons 6-9 years after disease.

	CF Titer								
	1:512	256	128	64	32	16	8	4	0+)
Number of persons	0	0	0	4	5	8	16	5	1

+) Serum at dilution 1:4 negative.

Table 2: CF antibody of 32 persons one year after infection.

	CF Titer								
	1:512	256	128	64	32	16	8	4	0
Number of persons	0	1	0	3	8	3	11	6	0

Table 3: CF antibody of 34 patients during convalescence.

	CF Titer					
	1:512	256	128	64	32	16
Number of persons	1	7	13	11	2	0

Table 4: HI antibody of 42 patients 6-9 years after disease.

	HI Titer									
	1:1024o	512o	256o	128o	64o	32o	16o	8o	4o	2o
Number of persons	0	0	0	1	2	6	13	13	6	1

Table 5: HI antibody of 39 patients in year of disease.

	HI Titer								
	1:1024o	512o	256o	128o	64o	32o	16o	8o	4o
Number of persons	1	1	1	7	12	9	6	2	0

Table 6: Patterns of CF and HI antibody during convalescence and 7-8 years after overt TBE.

Patient year	Test	Titer Year of disease	One year later	In 1967
W.B. 1959	CF	512	32	32
	HI	1o24o	n.d.	32o
K.M. 1959	CF	128	4	4
	HI	256o	n.d.	8o
G.E. 1959	CF	64	8	8
	HI	64o	n.d.	8o
Sch.H. 1959	CF	128	8	8
	HI	64o	n.d.	16o
St.H.P. 1959	CF	64	16	16
	HI	64o	n.d.	8o
E.F. 196o	CF	256	8	8
	HI	128o	n.d.	16o
K.P. 196o	CF	256	64	64
	HI	512o	n.d.	8o
G.J. 196o	CF	256	64	64
	HI	32o	n.d.	8o

Table 6: (Continuation)

Patient year	Test	Year of disease	One year later	In 1967
E.A. 1960	CF	128	8	8
	HI	1280	n.d.	40
M.F. 1960	CF	128	32	16
	HI	640	n.d.	160
St.R.1960	CF	128	16	16
	HI	640	n.d.	160
R.F. 1960	CF	128	8	4
	HI	640	n.d.	40
F.H. 1960	CF	64	8	8
	HI	160	n.d.	40
Sch.R. 1960	CF	64	8	8
	HI	640	n.d.	80
F.J.1960	CF	32	8	8
	HI	160	n.d.	40

n.d.: not done

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(7) Attempts at attenuation of TBE virus

It was stated in last year's report (1) that strain V 175 of TBE virus had reached a considerable degree of attenuation after continuous passage at 34° C in chick embryo lung cells. This work was carried on with the hope that a strain could be obtained which would not kill white mice after peripheral application but would still provoke an immune response in them.

By now this strain has become completely attenuated since mice succumb to infection only after intracerebral injection of the virus. The average survival time of the mice is markedly prolonged as compared with mice infected with low level passage of the virus. However, after peripheral application of the attenuated strain no immunity in mice was developed. They invariably died when challenged with virulent strains.

This obvious overattenuation of our virus seems to be a phenomenon similar to the one (2) Hammon et al. observed with Japanese encephalitis virus when it was grown at low temperature.

Since attenuated strain 175 failed to protect mice against challenge infection, further studies along this line do not seem to be promising. Nevertheless it appears to be of interest to compare the histological picture in mice caused by this virus with the lesions usually observed in the brains after infections with virulent TBE virus.

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SEROLOGICAL INVESTIGATIONS ON PATIENTS

Diagnostic work on suspected cases of TBE was performed with the methods repeatedly described in previous reports.

Hospital of Neunkirchen: From January through December 1966 a total of 85 patients was hospitalized with the clinical symptoms of a viral encephalitis (Table 1). TBE could be diagnosed in the laboratory in 21 cases, 1 case was presumably TBE and in 63 cases this disease could be ruled out. The monthly distribution of TBE cases was somewhat different from other years because the peak incidence of the disease in summer was not so pronounced as usual. The number of patients who had contracted TBE was slightly higher than in 1965 (15 cases), but the rate of TBE among nonbacterial infections of the CNS was surprisingly low. This is due to an epidemic of meningitis serosa caused by ECHO virus type 9 which occurred in Austria in the later part of summer and in autumn.

Other hospitals in Austria: From January through December specimens from 674 patients were received for laboratory investigations. The results, excluding those obtained in Neunkirchen, are reported in Table 2.

The number of 115 TBE cases (+ 21 cases in Neunkirchen) is almost the same as in 1965, when 129 cases of TBE were diagnosed in our laboratory.

From the studies which by now had been carried on for several years it is striking that the figures found in different years have been quite similar. This presumably reflects the stability of the TBE virus foci in Austria.

Hospitals in Western Germany: The results of investigations done with sera received from various hospitals in Western Germany are summarized in Table 3. Three TBE cases were again found among patients hospitalized in Freiburg/Br. and two among patients hospitalized in Munich. To our knowledge these persons had visited either the Black Forest or the Bavarian woods before the onset of illness. This

again provides strong evidence for the presence of TBE virus foci in these forests.

Table 1: Rate and monthly distribution of TBE among 85 patients with nonbacterial infections of the CNS in the hospital of Neunkirchen.

Month	Confirmed TBE	Possibly TBE	Not TBE
January	0	0	0
February	0	0	0
March	0	0	2
April	0	0	1
May	2	0	2
June	6	0	5
July	2	0	10
August	4	0	18
September	3	1	14
October	4	0	9
November	0	0	1
December	0	0	1
	21	1	63

Table 2: Cases of TBE among 674 patients hospitalized in Austria with nonbacterial infections of the CNS.

Province	Confirmed TBE	Possibly TBE	Not TBE
Vienna	34	5	172
Lower Austria	41+)	5+)	251+)
Upper Austria	14	1	47
Burgenland	15	3	32
Salzburg	1	-	26
Carinthia	6	-	5
Styria	4	-	11
The Tyrols	-	-	1
Total	115	14	545

+) These figures do not include the results from Neunkirchen.

Table 3: Results of serological investigations on 98 suspected cases of TBE in Western Germany.

Town	TBE	Not TBE
München	2	29
Freiburg/Br.	3	24
Gießen		2
Erlangen		1
Nürnberg		
Bamberg		1
Frankfurt/Main		16
Stuttgart		5
Hannover		1
Weingarten/Württ.		2
Ravensburg		4
Reutlingen		1
Total	5	93

PREPARATION OF A BATCH OF HYPERIMMUNEGLOBULIN AGAINST TBE VIRUS

In coöperation with the Austrian Red Cross, sera were collected from TBE patients shortly after convalescence. Plasma samples with a titer in the complement-fixation test of at least 1:64 were pooled. Twelve liters of plasma were obtained. The pool was processed giving a yield of 800 ml of a 16 % hyperimmuneglobulin solution. The titer of this batch in the hemagglutination-inhibition test with 8 units of antigen is 1:1280 per 0.1 ml.

TBE usually exhibits a biphasic course with a non-characteristic febrile illness (Phase I) 1-2 weeks after tick bite. At this time, virus can be demonstrated in the blood. This viremic stage is interrupted by the formation of antibodies and then (between 5-15 days after the onset of Phase I) the patient develops the symptoms of Meningoencephalitis (Phase II). Since at the beginning of Phase II neutralizing and hemagglutination-inhibiting antibodies can invariably be detected in the patient's blood, the application of hyperimmuneglobulin at this stage is useless.

It is, therefore, planned to conduct a field study with the purpose of getting some information on whether or not hyperimmuneglobulin can be effective when given during Phase I of the disease. It is hoped that if injected early enough, the antibodies can prevent the virus from attacking the central nervous system.

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13. ABSTRACT

In field studies 27 virus strains were isolated from ticks. A focus of TBE virus was found considerably further west than any other focus previously detected. Sera of small mammals showed a low incidence and sera of game showed a high incidence of antibodies to TBE virus. Virus strains could be rapidly identified with fluorescent antibodies. The innate resistance to TBE virus of Mus musculus spicilegus is probably due to a single dominant gene. The mouse brain lipid capable of inhibiting hemagglutination of TBE virus is most probably phosphatydil-inositol-diphosphate. Its full action requires the presence of auxiliary lipids containing a trimethyl-ammonium group. Preliminary results indicate that the lipid can also inhibit the virus adsorption onto tissue culture cells. In sera from both the acute stage of TBE and from convalescence complement-fixing antibodies were predominantly found in the 7 S γ globulin fraction. From 39 persons 38 still had complement-fixing and all had hemagglutination-inhibiting antibodies 6-9 years after overt TBE. Continuous propagation of a TBE virus strain led to over-attenuation of the virus. In 1966, 136 TBE cases from Austria and 5 cases from Western Germany were diagnosed.

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